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ANTIMICROBIAL RESISTANCE

Occurrence of methicillin-resistant and -susceptible *Staphylococcus aureus* within a single colony contributing to MRSA mis-identification

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Many methods have been described for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA), but the homogeneous or heterogeneous expression of methicillin resistance affects the reliability of those methods. This study demonstrates that close association between methicillin-susceptible *S. aureus* (MSSA) and MRSA strains in the host colonisation site can present additional problems for the detection of MRSA in clinical laboratories, which may contribute to failure in the control of MRSA infection in hospital. Worse, this association may also account for the emergence of MRSA during antibiotic therapy.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important cause of nosocomial infections and has become endemic in hospitals world-wide. Control of MRSA infection and colonisation is difficult and may result in serious clinical and managerial problems [1–4].

The *mecA* gene is the determinant of methicillin resistance and encodes for the penicillin-binding protein 2a or 2′. This 78-kDa membrane protein has low affinity for β-lactam antibiotics [5]. Heterogeneous MRSA strains show a great degree of variation in the phenotypic expression of methicillin resistance [6] and a number of genes seems to be involved [7, 8].

MRSA clinical isolates can be divided into four phenotypic expression classes (classes 1–4) by population analysis profile experiments [9]. In the heterogeneous phenotype only a small proportion of the cells in the culture express resistance at high level while the great majority of the population have MIC values similar to those observed for susceptible *S. aureus* strains (MSSA). These profiles were shown to be stable *in vitro* [9] and *in vivo* [6]. The occurrence of these complex phenotypes among MRSA clinical isolates contributes to mis-identification of MRSA [10–11]. In MRSA class 1 phenotype, the methicillin MICs for the majority of bacteria vary from 1.5 to 3.0 mg/L and only 10^{-8}–10^{-6} of the population express higher MIC values (50–100 mg/L). A similar phenomenon has been demonstrated in class 2 strains. The MIC for the majority of cells varied from 5 to 25 mg/L and only a small part of the population (10^{-5}–10^{-4}) gave higher MIC values (*c.* 200–400 mg/L). MRSA classes 3 and 4 have very high MIC values for methicillin – for the majority the MIC is >400 mg/L. Thus, the identification of the methicillin resistance among *S. aureus* strains belonging to class 3 or 4 phenotype does not present a problem for MRSA detection in clinical laboratories [12]. However, because the MICs of methicillin for the majority of the population in classes 1 and 2 may be as low as 1.5–5.0 mg/L, MRSA giving these profiles may not be readily detected by routine methods (a methicillin MIC for truly susceptible *S. aureus* is 0.5–1.0 mg/L and 2.0–8.0 mg/L for borderline strains of *S. aureus*) [12].

This study describes another problem that can affect MRSA identification and may contribute to the emergence of methicillin resistance during antibiotic therapy. MRSA strains from the anterior nares of human carriers were examined for the presence of a

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mixed population with MSSA strains within a single colony.

Materials and methods

Strains

Nasal swabs were obtained from 37 students (who were attending second year in the Medical School) and healthy workers (from Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, RJ, Brazil). The medical students and workers in this study were not attending classes or working in a hospital. However, they had frequent contact with clinicians and health-care personnel from the University Hospital, located *c.* 100 m from the Centro de Ciências da Saúde. Nasal swabs were also obtained from 31 health-care workers and from 10 patients in the intensive care unit of Hospital Samaritanos, Rio de Janeiro. *S. aureus* isolates were identified by routine methods [11]. For preparation of stocks, *S. aureus* cultures received in agar slants from the hospital or obtained from nasal swabs of medical students were plated on trypticase soy agar (TSA). One isolated colony of each culture was grown to stationary phase in trypticase soy broth (TSB) and stored at −70°C with sterile glycerol (final concentration 12% v/v). These stocks were called original stocks and the cultures obtained directly from those stocks were designated as original cultures. *S. aureus* strains representing each class of resistance (classes 1, 2, 3 and 4) were obtained from Dr Alexander Tomasz [9].

Methicillin agar screen

A 100-μl sample of each original stock was inoculated into 2 ml of TSB and incubated at 37°C in a shaking device to ensure vigorous aeration and a high bacterial cell concentration (*c.* 10^9-10^{10} cfu/ml). The cultures (100-μl portions) were plated on TSA containing methicillin 25 mg/L. Resistance was confirmed by the presence of colonies on the surface of methicillin plates after incubation for 24 h at 37°C, as described previously [12]. This test gives 100% correlation with a specific *mecA* probe, when performed at either 35°C or 37°C [12].

Population analysis profile (PAP)

Overnight cultures (10^9-10^{10} cfu/ml) were plated at different dilutions on TSA containing methicillin at concentrations of 0.75–800 mg/L and on methicillin-free TSA. Cfu were counted after incubation at 37°C for 48 h [6].

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described previously [2, 13], after DNA fragmentation with *Sma*I, in a CHEF DR III apparatus (BioRad). Methodologies used for staining and photographing the gels have been described previously [14]. Bacterial clones were defined as proposed by Tenover *et al.* [15].

Detection of the mecA gene with a specific probe

*Sma*I-fragmented genomic DNA samples were transferred from the pulsed-field gel to a nylon membrane with a vacuum-blot apparatus as recommended by the manufacturer (Pharmacia). The procedure to obtain the labelled probe with the Enhanced Chemiluminescence (ECL) Gene Labelling and Detection System (Amersham) was as described previously [2]. The DNA probe used was a *Pst*I-*Xba*I fragment of the *mecA* gene cloned into pTZ219 [16].

Disk diffusion test

This test was done as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [17]. Briefly, an overnight culture was diluted to 10^7-10^8 cfu/ml and evenly spread on a Muller-Hinton agar plate. The disks were applied and the plate was incubated at 35°C for 24 h.

Broth macro-dilution

The broth macro-dilution test was performed in Mueller-Hinton broth according to the guidelines of the NCCLS [18]. Oxacillin was added at final concentrations of 0.25–32 mg/L and the tubes were incubated at 35°C.

Combined oxacillin and methicillin agar screen

Mueller-Hinton agar was supplemented with oxacillin 6 mg/L or methicillin 10 mg/L and NaCl 4% w/v (final concentration). The inoculum was prepared as recommended by the NCCLS guidelines [18]. After incubation at 35°C for 24 h, MRSA should grow on these plates, whereas borderline-resistant and susceptible strains should fail to grow under these conditions.

Results

Population analysis profiles of MRSA isolates

Six isolates of MRSA were cultured from the noses of 37 students and workers from Universidade Federal do Rio de Janeiro on methicillin 25 mg/L screening plates. All the isolates in the original cultures were initially classed as heterogeneous class 1 or 2 MRSA by PAPs. However, the PAPs of different isolated colonies, for all six MRSA cultures obtained, varied considerably from class 1, 2 and 3 to susceptible, after one or two passages of the original culture on TSA without antibiotic (Fig. 1).

In an effort to explain this phenomenon, the liquid culture obtained from a susceptible isolated colony,

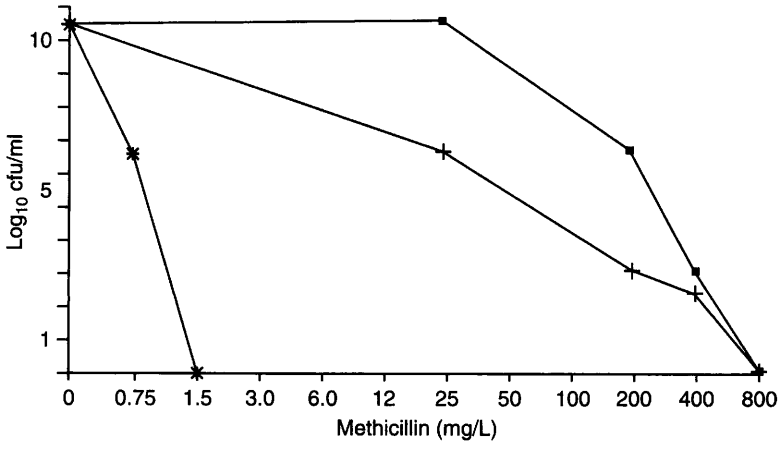

Fig. 1. Population analysis profile. **1**, (■) MRSA clone obtained from the original (mixed) culture O1. **2**, (+) The original culture O1. **3**, (*) MSSA clone obtained from the original (mixed) culture O1.

was purified through 10 serial passages on TSA plates without antibiotic. After that, the PAPs were repeated and the susceptibility pattern was confirmed for all 10 colonies examined. All the methicillin-susceptible strains purified were susceptible to most of the drugs tested in (Table 1).

The same purification step on TSA plates was performed for resistant colonies that grew on methicillin 25 mg/L plates. The PAPs of 10 isolated resistant colonies showed a high level of methicillin resistance and a typical class 3 phenotype. The purified resistant strains showed multiresistance in the disk diffusion test, contrasting with the results obtained for purified methicillin-susceptible colonies (Table 1). These results indicated that there was a mixed population of methicillin-susceptible and -resistant strains within a single colony.

PFGE patterns from each MRSA-MSSA pair isolated within single colonies

To study the mechanism involved in this close association of susceptible and resistant strains, genomic DNA was prepared from individual, purified, susceptible and resistant *S. aureus*. After treatment with the endonuclease *Sma*I, the restriction fragment length polymorphism (RFLP) of the chromosomal DNA from each MRSA-MSSA pair showed that, for the five pairs analysed, the susceptible strains gave a pattern very distinct from the methicillin-resistant isolates, with more than six different pulsed-field bands (Fig. 2). These results indicate that each of the pair of susceptible and resistant strains isolated within a single colony clearly belonged to a different clone.

The presence of the *mecA* gene in the resistant

Table 1. Disk diffusion test of the MSSA and MRSA strains isolated in close association from the same colonisation site

	Strain*										
Antimicrobial drug†	O1R	O1S	O2R	O2S	O3R	O3S	O4R	O5R	O5S	O6R	O6S
Cip	S	S	S	S	S	S	S	S	S	R	S
Vc	S	S	S	S	S	S	S	S	S	S	S
Tc	R	R	S	S	R	S	R	R	S	R	S
Cf	R	S	R	S	R	S	R	R	S	R	S
Gn	R	S	R	S	R	S	R	R	S	R	S
Co	R	S	R	S	R	S	R	R	S	R	S
Sft	R	S	S	S	R	S	R	R	S	R	S
Cl	S	S	R	S	S	S	R	R	S	R	S
Et	R	S	R	S	R	S	R	R	S	R	S
Pn	R	R	R	S	R	R	R	R	R	R	S

R, resistant; S, susceptible.

*S. *aureus* strains O1R, O2R, O3R, O4R, O5R and O6R were methicillin-resistant isolates obtained from original (mixed) cultures after growth on methicillin 25 mg/L agar plates. The susceptible strains isolated from mixed cultures (except for O4S) were obtained after passages of the original cultures on trypticase soy agar without antibiotic.

†Cip, ciprofloxacin (5 μg); Vc, vancomycin (30 μg); Tc, tetracycline (30 μg); Cf, cephalothin (30 μg); Gn, gentamicin (10 μg); Co, chloramphenicol (30 μg); Sft, sulphamethoxazole-trimethoprim (23.75–1.25 μg); Cl, clindamycin (2 μg); Et, erythromycin (15 μg); Pn, penicillin (10 U).

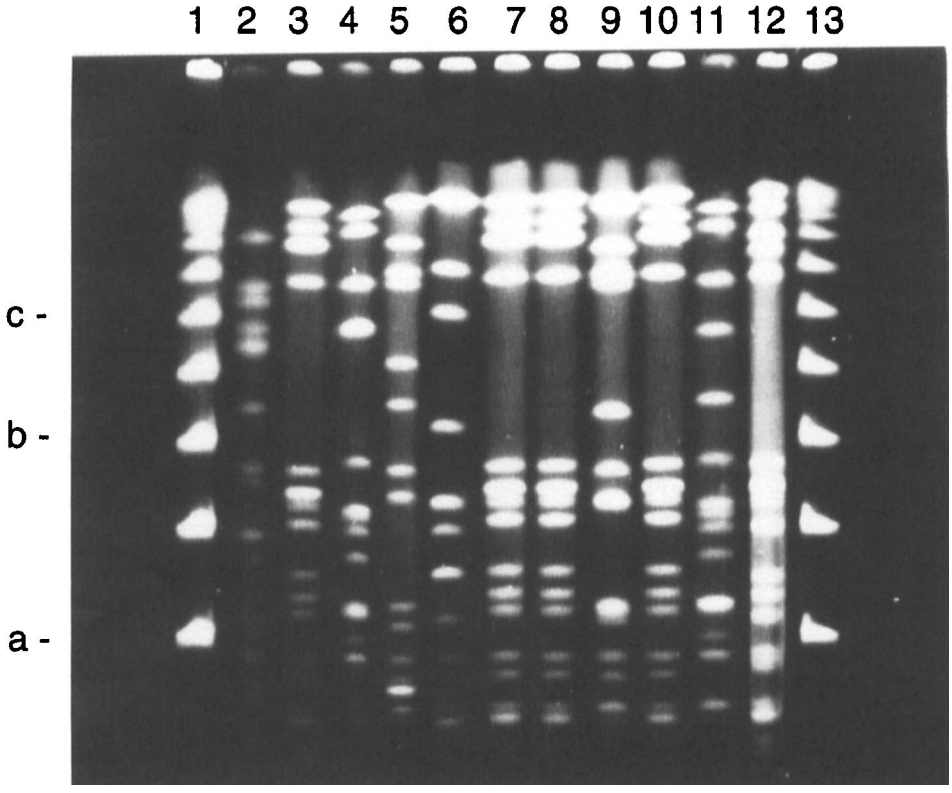

Fig. 2. PFGE patterns of the MSSA and MRSA strains isolated from original (mixed) cultures **1**, λ ladder: **a**, 48.5 kb; **b**, 145.5 kb, **c**, 242.5 kb. **2**, MSSA clone from the original (mixed) culture O1. **3**, MRSA clone from the original (mixed) culture O1. **4**, MSSA clone from the original (mixed) culture O2. **5**, MRSA clone from the original (mixed) culture O2. **6**, MSSA clone from the original (mixed) culture O3. **7**, MRSA clone from the original (mixed) culture O3. **8**, MRSA clone from the original (mixed) culture O4. **9**, MSSA clone from the original (mixed) culture O5. **10**, MRSA clone from the original (mixed) culture O5. **11**, MSSA clone from the original (mixed) culture O6. **12**, MRSA clone from the original (mixed) culture O6. **13**, λ ladder: **a**, 48.5 kb; **b**, 145.5 kb; **c**, 242.5 kb.

isolates that grew on methicillin 25 mg/L agar plates was confirmed with a specific DNA probe. As shown in Fig. 3, the purified class 3 heterogeneous strains, isolated from the mixed cultures, hybridised with the *mecA* probe. As expected, all five susceptible strains, purified after 10 passages on TSA plates without antibiotic, did not produce signals after hybridisation experiments with the specific *mecA* probe, except for the isolate O4S that could not be totally separated from the MRSA strains and therefore was not used in this analysis. It is also possible that the O4 original culture was a truly heterogeneous resistant strain in mixed population with a homogeneous MRSA isolate.

Evaluation of the routine methods recommended by the NCCLS to detect MRSA strains in a mixed population with MSSA

The MSSA strain always predominated in this mixed population (10^7–10^9 cfu/ml) and the MRSA concentration was as low as 10–10^3 cfu/ml. The implication of this was that when the original (mixed) cultures were used for the detection of MRSA, some of the routine methods recommended by the NCCLS were not appropriate for the identification of MRSA strains in these circumstances, particularly the disk diffusion test and broth macro-dilution. Both tests completely failed to detect MRSA in the original cultures. Only the screening tests were able to identify MRSA in these mixed cultures (Table 2). Furthermore, methicillin 25 mg/L agar enabled the isolation of a higher number of cfu/plate in most of the cases.

Clinical significance of mixed MRSA-MSSA cultures

To analyse the clinical significance of this phenomenon, the presence of this association of MRSA-MSSA within a single colony was sought in 10 original cultures of *S. aureus* obtained from patients from the intensive care unit of the Hospital Samaritano; *S. aureus* strains obtained from the noses of health-care workers from the same hospital were also studied. Of six MRSA nasal cultures from 31 health-care personnel, four yielded MRSA-MSSA mixtures. The MSSA strains isolated were resistant only to penicillin whereas the MRSA showed susceptibility to vancomycin only. Tenover *et al.* [19], comparing 13 different phenotypic and molecular methods of typing isolates of *S. aureus*, showed that changes in the zone sizes around disks for two or more antimicrobial agents allowed differentiation of strains. Furthermore, the fact that these MSSA isolates lacked both chromosome- and plasmid-associated resistance genes that were present in the

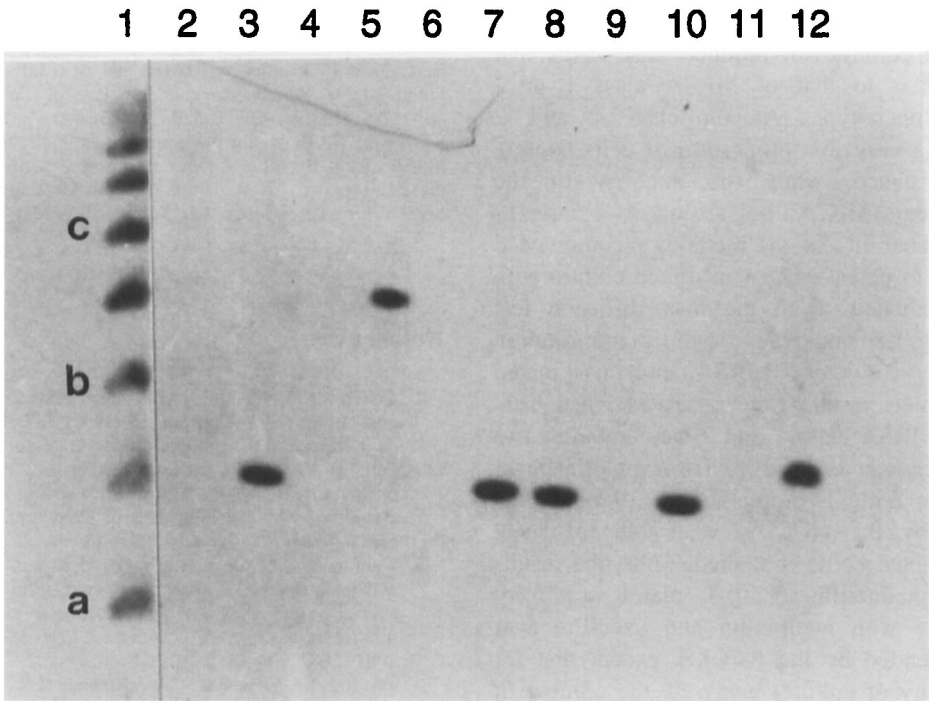

Fig. 3. After PFGE the chromosomal DNA of the MRSA and MSSA strains were blotted on a nylon membrane with a *mecA* specific probe [11]. **1**, λ ladder: **a**, 48.5 kb; **b**, 145.5 kb; **c**, 242.5 kb. **2**, MSSA clone from the original (mixed) culture O1. **3**, MRSA clone from the original (mixed) culture O1. **4**, MSSA clone from the original (mixed) culture O2. **5**, MRSA clone from the original (mixed) culture O2. **6**, MSSA clone from the original (mixed) culture O3. **7**, MRSA clone from the original (mixed) culture O3. **8**, MRSA clone from the original (mixed) culture O4. **9**, MSSA clone from the original (mixed) culture O5. **10**, MRSA clone from the original (mixed) culture O5. **11**, MSSA clone from the original (mixed) culture O6. **12**, MRSA clone from the original (mixed) culture O6. **13**, λ ladder: **a**, 48.5 kb; **b**, 145.5 kb; **c**, 242.5 kb.

Table 2. Reliability of recommended tests for MRSA detection in MRSA-MSSA original (mixed) culture isolated from carriers

	Number of cfu/plate by recommended methods				
Original (mixed) culture	25 Met† (cfu/plate)	10 Met (cfu/plate)	6 Oxa (cfu/plate)	Oxa disk‡ (mm)	MIC§ (mg/L)
O1	>1000	140	114	21 (S)	2 (S)
O2	>1000	620	480	13 (I)	1 (S)
O3	30	5	4	15 (S)	0.5 (S)
O4	900	620	480	15 (S)	2 (S)
O5	82	1	2	16 (S)	2 (S)
O6	80	81	131	16 (S)	2 (S)

*Original (mixed) culture was a liquid culture from a glycerol-containing stock obtained from a single colony.
†25 Met, 10 Met and 6 Oxa were agar plates containing methicillin, 25 mg/L [12], 10 mg/L and 6 mg/L, respectively. The inoculum added to the plate was *c.* 10^8-10^9 cfu/plate for 25 Met and 10^6-10^7 cfu/plate for 10 Met and 6 Oxa. NaCl to a final concentration of 4% was added to the 10 Met and 6 Oxa plates, as recommended by the NCCLS [18].
‡The oxacillin 1 μg disk was used. Isolated colonies (ranging from 0 to 100) were verified inside the halo zone, when the original (mixed) cultures were tested.
§MIC, the minimal inhibitory concentration was determined with liquid medium, as recommended by the NCCLS [18].

MRSA strains clearly indicated that MRSA and MSSA in these pairs also belonged to distinct clonal origins. In contrast, mixed cultures were not observed in MRSA-positive cultures from clinical material of infected or colonised patients (data not shown).

Discussion

The use of an agar screen (methicillin 25 mg/L) and a high inoculum (10^9-10^{10} cfu/ml) made it possible to detect a close association between distinct MRSA and MSSA clones within a single colony. MRSA in this mixed culture were present in lower numbers (10–10^3 cfu/ml) than MSSA (10^7-10^9 cfu/ml). One possible explanation for the predominance of MSSA is the absence of selective pressure in the study population, as the students and the healthy workers from the Centro de Ciências da Saúde and the health-care personnel from Hospital Samaritano were not on antibiotic therapy. The use of antimicrobial therapy by the hospitalised patients studied may explain why MRSA was not observed in association with MSSA when clinical samples and nasal swabs were examined.

The study verified that mixed culture MRSA-MSSA, within a single colony, can produce false PAPs that were very similar to that of MRSA class 1 or 2 heterogeneous phenotype. Among class 1 and 2 MRSA strains, a very low proportion of cells express methicillin resistance, while the majority of the population presents MIC values as low as 1.5 mg/L. Therefore the reliability of the methods recommended by the NCCLS to detect MRSA in mixed culture with MSSA was evaluated. Both the disk diffusion test (oxacillin 1 mg/L) and the broth macro-dilution method failed to detect MRSA in this mixed population. Similar results were observed when truly heterogeneous MRSA class 1 and 2 were submitted to those recommended tests [20]. However, the agar screen (oxacillin 6 mg/L or methicillin 10 mg/L), as recommended by the NCCLS, was able to detect MRSA in all mixed cultures studied. Thus, the results obtained with methicillin 25 mg/L plates were very similar to those with methicillin and oxacillin agar plates, recommended by the NCCLS, except that for the great majority of cultures analysed, the number of methicillin-resistant colonies per plate was higher when methicillin 25 mg/L agar was used. This may be explained by the high bacterial inoculum used in this last test (10^8–10^9 cfu/plate).

The emergence of methicillin resistance during antibiotic therapy directed to *S. aureus* infection or any other bacterial disease might be another consequence of this MRSA-MSSA association. As most MRSA strains are multi-resistant [4, 11], many of the antibiotics used in therapy (except vancomycin) may select for methicillin-resistant staphylococci [21].

Previous studies showed that MRSA and MSSA were isolated from cultures derived from the same patients [3, 22]. However, in those studies the MRSA clones were genetically similar to the MSSA clones. The detection of the association of distinct MRSA-MSSA clones, within a single colony, colonising health-care workers from Hospital Samaritano, Rio de Janeiro, and the recent report of a similar association of MRSA-MSSA by an independent research group [23] indicate that the phenomenon presented here is not a simple technical artefact. The existence of this bacterial association interfered with the detection of MRSA and thus it may contribute to failures in the control of MRSA transmission in hospitals caused by the presence of 'silent' MRSA carriers.

In view of the results presented here the use of heavily inoculated methicillin 25 mg/L agar is strongly recommended for screening for MRSA carriers. In addition, serial passages of the primary culture on media without antibiotic should be avoided, in order to improve the detection of MRSA.

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